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STUDIES ON THE INHALATION TOXICITY
OF DYES PRESENT IN COLORED
SMOKE MUNITIONS
FINAL REPORT FOR PHASE III STUDIES:
FOUR-WEEK INHALATION EXPOSURES OF RATS TO DYE AEROSOLS

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21. ABSTRACT (Continue on reverse side if necessary and identify by block number) The USAMBRDL has an interest in the potential inhalation toxicity of yellow dye (SY) and a yellow/green dye mixture (SY/SG) used in colored smoke munitions. Rats were exposed by inhalation to aerosols of SY or SY/SG generated in a respirable particle size range for 6 hr/day, 5 days/week for 4 weeks. Nominal exposure concentrations selected for both dye exposures were 0, 10, 50, and 250 mg/m ³ . Animals exposed to the highest concentration of SY (230 mg/m ³) showed only a slight decrease in weight gain (8 percent).		

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and mild respiratory function changes, with no observable histopathological lesions. Exposure to the lower concentrations of SY dye elicited no observed response. Animals exposed to the highest level of SY/SG dye (210 mg/m³) displayed signs of pulmonary inflammation with histopathological evidence of mild Type II pulmonary epithelial cell hyperplasia and proliferation of foamy alveolar macrophages. Some of the animals exposed to the medium concentration of SY/SG dye mixture (49 mg/m³) showed similar signs. Thus, the lowest toxic exposure concentration of SY dye is ≥ 230 mg/m³ and that of SY/SG dye is ≥ 50 mg/m³ under the exposure regime used. Originator

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EXECUTIVE SUMMARY

The USAMBRDL has an interest in the potential inhalation toxicity of yellow dye (SY) and a yellow/green dye mixture (SY/SG) used in colored smoke munitions. The chemical name of the major component of the yellow dye is 2-(2'quinoly1)-1,3-indandione (QI) and the major component of the green dye is 1,4-di-p-toluidinoanthraquinone (TA). SY contains only QI, while the SY/SG dye mixture contains 30 percent QI and 70 percent TA. To test the inhalation toxicity of these materials, exposure atmospheres of SY or SY/SG were generated in a respirable particle size range and used for inhalation exposures of F344 rats at three concentrations for 6 hr/day, 5 days/week for 4 weeks. The purpose of these studies was to determine the lowest air concentration for each dye material that would produce pathological changes. For these studies, the biological indicators of toxicity measured were gross clinical observations, changes in body weight gain, respiratory function measurements, biochemical and cellular analysis of bronchoalveolar lavage fluid, lung tissue enzyme and connective tissue biochemistry, blood and serum chemistries, skin sensitization, and histopathology of selected tissues. This report describes the results of these investigations.

In the four-week inhalation exposure to SY dye, rats were exposed to aerosols of SY dye at average concentrations of 10 ± 5 , 51 ± 10 or 230 ± 30 mg/m³ ($\bar{X} \pm SD$). The average particle size of these aerosols ranged from 3.1 to 4.4 μ m (mass median aerodynamic diameter) with a geometric standard deviation (σ_g) of approximately 2.0. After exposures, it was found that very little SY dye (QI) was retained in the lungs, indicating the SY dye was rapidly cleared. The body weights of the animals exposed to the highest

concentration averaged 8 percent less than controls ($p \leq 0.05$) at the end of the exposure. Respiratory function measurements were made on the control and high dose groups only and showed mild respiratory function changes consisting of reduced elastic recoil, increased resting lung volumes and reduced expiratory flowrates in the exposed rats.

Rats were exposed to aerosols of SY/SG dye mixture having average concentrations of 11 ± 5 , 49 ± 11 or $210 \pm 50 \text{ mg/m}^3$ ($\bar{X} \pm \text{SD}$). The average particle size of these aerosols ranged from 3.1 to 4.9 μm (mass median aerodynamic diameter) with a σ_g of approximately 2.0. As with the studies on SY dye exposures, it was found that the Q1 component of the SY/SG dye mixture cleared from lungs at a very rapid rate. However, the SG (TA) portion of the SY/SG dye mixture was retained in lungs for a much longer time. Rats exposed to the highest concentration of SY/SG dye mixture had a significantly lower body weight after four weeks of exposure than controls, approximately 7 percent less. Respiratory function measurements in high level exposed animals indicated a reduction of gas exchange efficiency and airflow obstruction. Analysis of bronchoalveolar lavage fluid showed an influx of neutrophils and increased enzyme activities indicating a mild pulmonary inflammatory response occurred in high level SY/SG exposed rats. Increased neutrophils were also seen in lavage fluid from the medium dose animals. Exposure-related histopathological lesions consisted of minimal to slight Type II pulmonary epithelial cell hyperplasia and proliferation of foamy alveolar macrophages in rats exposed to the highest concentration of SY/SG dye. This mild response was also seen in some of the medium dose animals. No other adverse effects were observed in the various parameters measured in exposed animals as compared to control rats.

In summary, no histopathological lesions were observed in rats exposed to up to 230 mg/m^3 of SY dye for 20 days over a 4 week period. An 8 percent difference in body weight and mild respiratory function changes were the only toxic signs observed. In rats exposed to the SY/SG dye mixture, an exposure concentration of 210 mg/m^3 was sufficient to induce an inflammatory response in the lung. Thus, the lowest toxic exposure concentration of SY is $\geq 230 \text{ mg/m}^3$ and of SY/SG is $\geq 50 \text{ mg/m}^3$ under the above exposure conditions. It is apparent that the SY/SG dye mixture is more toxic to the lung than is the SY dye. This is at least partially due to the longer retention time in the lung for the SG portion of the dye mixture as compared to the SY.

PREFACE

The authors acknowledge the outstanding contributions of all members of the Inhalation Toxicology Research Institute, without whose help these studies could not have been completed. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Users of Laboratory Animals," prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 80-23, Revised 1980). This research was supported by the U. S. Army Medical Research and Development Command under a Memorandum of Understanding Agreement No. AT(29-2)-2138/3807 with the Lovelace Inhalation Toxicology Research Institute, which is operated for the U. S. Department of Energy under DOE Contract No. DE-AC04-76EVO1013.

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INTRODUCTION

The U. S. Army needs to obtain information on the inhalation toxicity of the dyes present in colored smoke munitions. The major concern is for the health of munition production workers who could be exposed to fine dusts containing the dyes during production of the smoke munition. In the project, "Studies on the Inhalation Toxicity of Dyes Present in Colored Smoke Munitions (Project Order No. 83PP3807)," the Lovelace Inhalation Toxicology Research Institute (ITRI) is studying the inhalation toxicity of two dye materials: a yellow dye (SY) and a yellow/green dye mix (SY/SG). The chemical name of the yellow dye is 2-(2'-quinoly1)-1,3-indandione (QI). Various synonyms used for the dye include C.I. solvent yellow 33, C.I. 47 000 and D & C yellow No. 11. The green dye is 1,4-di-p-toluidinanthraquinone (TA) and has been called C.I. solvent green 3, C.I. 61565 and D & C Green No. 6. The yellow/green dye mix contains approximately 30 percent yellow dye and 70 percent green dye. The smoke munitions will contain 42 percent by weight of the dyes. The munition also contains potassium chlorate, magnesium carbonate and sucrose.

The work is being conducted in four phases. Phase I includes standardization of methods for generation of aerosols of the test materials and physical/chemical characterization of the aerosols. Phase II consists of range-finding experiments to determine acute toxic effects from exposure to high concentrations of the dyes and to select exposure concentrations for the next two phases of the study. In Phase III, four-week exposures of animals to varying concentrations of the dyes will be used to determine the lowest exposure concentration that will produce pathological changes. Phase IV will

be a 90-day subchronic study to determine a no-observable-adverse effects level (NOAEL) of exposure.

This is a final report of the work completed in Phase III of these studies.

FOUR-WEEK INHALATION EXPOSURE TO SY DYE

Nominal exposure concentrations selected for this study were 0, 10, 50, and 250 mg/m³ given for 6 hours/day, 5 days/week, for 4 weeks. These concentrations were selected on the basis of several criteria. In the range-finding studies (Phase II),¹⁻³ we had no mortality and saw no life-threatening lesions in tissues from rats exposed to 1300 mg SY/m³ for 6 hours per day for 5 days with a particle size of 5.6 µm (MMAD). We did detect alterations in the histology of the upper respiratory tract. In the lower exposure levels used for the 4-week exposure, the particle size would be smaller (3-5 µm) resulting in a larger respirable fraction, so that a 120-hour exposure (6 hours/day, 5 days/week, for 4 weeks) to 250 mg/m³ was estimated to be roughly equivalent to the total dye exposure in the 30-hr range-finding study. Thus, at the high exposure level it was anticipated that the pathological alterations would be approximately equivalent to those seen in the range-finding studies without any expected mortality. The lower exposure level of 10 mg/m³ was selected because it is the current ACGIH nuisance dust threshold limit value (TLV).^{*} The intermediate level of 50 mg/m³ was selected because it is a factor of 5 between the high and low levels.

Statistical Analysis

Where possible, computer software packages such as BMDP or RSI were used for data handling and analysis. Standard tests applied to the experimental data were one-way analyses of variance, followed by tests for the equality of

^{*}The Threshold Limit Value - Time Weighted Average (TLV - TWA) is the time-weighted average concentration for a normal 8-hour workday and a 40-hour work week, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect.

means, such as simple t-tests, multiple comparison t-tests,⁴ or non-parametric Mann-Whitney U tests. Usually a confidence level of 0.05 was used, although in some cases significance was expressed in much smaller or much larger values.

Animals

Specific pathogen free, male and female Fischer-344 (F344/Crl Lov) rats, 15-20 weeks of age with an age range of ± 2 weeks, were used for this study. The rats were obtained from an existing colony raised at the Inhalation Toxicology Research Institute. At the age for experimental use, rats were moved to Hazleton 2000 exposure chambers in the Chronic Exposure Laboratory. The animals were acclimated in exposure chambers for at least 2 to 3 weeks before use to acclimate them to that environment and to ascertain their physical well being. After completion of the pre-exposure acclimatization period, the rats were randomly assigned to experimental groups by litter number and weight. Each rat was uniquely identified using ear tags. During these studies all animals were given food and water ad libitum except that food was withheld during exposure periods.

All rats were euthanized at the end of the studies by intraperitoneal injections of T-61 euthanasia solution. The inhalation exposure chambers were maintained at $75 \pm 3^\circ\text{F}$ and 35-70 percent relative humidity. This temperature was above what is normally found in an animal room ($72 \pm 2^\circ\text{F}$) in order to compensate for body heat loss due to chamber airflow. Chamber airflow rate, temperature, humidity, and pressure relative to the room were monitored throughout this study. Temperature and humidity measuring devices were calibrated and checked before initiation of the study.

Animal Exposures

Aerosols of SY dye material were generated using a Jet-O-Mizer air jet mill as previously described.^{5,6} Four Hazleton 2000 chambers containing 6 tiers of animal cages (10-16 rats per tier) were used for these whole-body inhalation exposure studies. Before the 4-week study began, all chamber exposure systems were run for 6 hours as a final check of the stability of the aerosol generation system. Results are shown in Table 1.

For these studies, 228 rats (114 males and 114 females) were entered into each exposure chamber in three groups, which were staggered by one day. Concentration and size characteristics of the exposure atmospheres for each of the 22 exposure days are shown in Table 2. While this table indicates a significant degree of variance in the daily concentration of SY dye, particularly at the lowest concentration, the fluctuations were never so great that the concentration overlapped the concentration of another exposure level. Thus, in terms of exposing animals to graded levels of SY dye, acceptable separation among low, medium and high level exposure groups was attained. Exposure group I was exposed on days 1-20, exposure group II on days 2-21, and exposure group III on days 3-22. Table 3 summarizes the average SY concentration experienced by each of these groups of animals. With three data sets that are the same, except for a day at each end of the exposure period, it is not surprising that no statistically significant differences were seen between the three entry groups at each exposure concentration. The SY aerosol size was measured with a Lovelace Multi-Jet Cascade Impactor each day during the first week of exposure and at intervals as shown in Table 2 thereafter. Table 4 shows the average mass median aerodynamic diameter and average geometric standard deviation of these

TABLE 1
Concentrations and Particle Size of SY Dye Aerosols

Concentration ^a (mg/m ³)	Coefficient of Variation (Percent)	MMAD ^b (μ m)	σ_g ^c
16 \pm 2, n = 9	13	3.0	2.0
57 \pm 11, n = 6	19	4.0	2.0
250 \pm 30, n = 9	12	4.6	2.0

^aSamples taken at evenly spaced intervals during 6-hour period
(mean \pm SD).

^bMMAD = mass median aerodynamic diameter (mean; n = 2).

^c σ_g = geometric standard deviation.

TABLE 2

Daily Exposure Chamber Concentration and
Particle Size of SY Dye Aerosols

	Low			Medium			High		
	Conc. (mg/m ³)	MMAD ^a (μm)	σ _g ^b	Conc. (mg/m ³)	MMAD (μm)	σ _g	Conc. (mg/m ³)	MMAD (μm)	σ _g
Day 1	19.9	3.4	2.0	48.9	4.6	2.1	239	5.8	1.7
2	15.7	3.1	2.0	48.5	3.1	3.0	297	4.0	2.1
3	13.9	3.1	2.2	63.8	3.2	2.0	167	3.5	2.0
4	15.7	2.9	2.1	46.5	3.2	2.0	291	4.8	1.9
5	10.6	3.0	2.0	44.0	2.9	1.9	237	3.7	2.0
6	6.8	---	---	49.8	---	---	232	4.6	2.0
7	17.7	3.6	2.1	44.7	3.7	2.1	242	4.4	1.9
8	5.8	3.1	2.2	52.3	4.0	2.2	221	4.4	2.0
9	5.5	---	---	54.2	3.5	1.9	203	---	---
10	9.5	2.7	2.0	58.5	---	---	217	---	---
11	7.5	---	---	82.8	3.7	1.9	216	---	---
12	5.7	---	---	48.0	---	---	198	4.8	1.9
13	7.9	---	---	35.7	---	---	234	---	---
14	6.2	3.0	2.4	48.7	---	---	222	---	---
15	6.3	---	---	36.1	---	---	232	---	---
16	10.3	3.6	2.3	51.9	---	---	220	---	---
17	10.0	---	---	44.5	---	---	290	---	---
18	3.6	---	---	44.4	---	---	254	4.3	1.8
19	8.7	---	---	48.6	3.4	2.2	231	---	---
20	9.6	---	---	62.5	---	---	254	---	---
21	15.3	---	---	60.8	---	---	190	---	---
22	16.1	---	---	53.0	---	---	252	---	---
\bar{X}	10	3.2	2.1	51	3.5	2.0	230	4.4	1.9
S.D.	±5	0.3	0.1	±10	0.5	0.1	±30	0.7	0.1

Daily chamber concentrations were determined by the mean of 6 to 8 filter samples taken at regular intervals during the day (High dose chamber, all days; first 6 days in Low and Medium dose chambers) or by one 6-hour filter sample (Low and Medium dose chambers, days 7-22). The average variance among the daily filter sample collected from the low, medium and high dose chambers was 32 percent, 9 percent, and 18 percent of the mean, respectively.

^aMMAD = mass median aerodynamic diameter.

^bσ_g = geometric standard deviation.

TABLE 3

Average SY Aerosol Concentrations Measured During the
4-Week Repeated Inhalation Exposure of Rats*

	Exposure Chamber		
	Low (10 mg/m ³)	Medium (50 mg/m ³)	High (250 mg/m ³)
Group I	9.8 ± 1.0	50.7 ± 2.3	235 ± 7
Group II	9.6 ± 0.9	51.3 ± 2.4	232 ± 7
Group III	9.6 ± 0.9	51.5 ± 2.4	230 ± 7

*Average Concentration of SY (mg/m³) ± SE (n = 20). Values were not found to be statistically different between the three animal entry groups at each exposure concentration using a multiple comparison t-test of the means (for the null hypothesis $p > 0.2$). Note, however, that the three data sets are highly dependent between each other because they differ only by a few data points.

TABLE 4
Average Particle Size of SY Aerosols Used in
the 4-Week Repeated Exposure of Rats

	SY Exposure Concentrations		
	Low	Medium	High
Group I*			
MMAD**	3.2 ± 0.3	3.5 ± 0.5	4.4 ± 0.7
σ_g ***	2.1 ± 0.1	2.0 ± 0.1	1.9 ± 0.1
Group II*			
MMAD	3.1 ± 0.3	3.4 ± 0.4	4.3 ± 0.5
σ_g	2.2 ± 0.1	2.0 ± 0.1	1.9 ± 0.1
Group III*			
MMAD	3.1 ± 0.3	3.5 ± 0.3	4.3 ± 0.5
σ_g	2.2 ± 0.1	2.0 ± 0.1	1.9 ± 0.1

*Multiple comparison t-tests between the 3 group means show no differences of statistical significance (for the null hypothesis $p > 0.2$). The same tests between the 3 exposure concentrations also show no significant differences (for the null hypothesis $p > 0.1$).

**Average Mass Median Aerodynamic Diameter ± SD (n = 10).

***Average Geometric Standard Deviation ± SD (n = 10).

aerosols with respect to the different entry groups of animals and at each exposure concentration. Again, no differences are evident between groups of animals or between exposure groups, however, there may be a systematic trend to higher particle diameters in higher SY exposure concentration groups. Also during these exposures, one filter per week from each exposure level was analyzed for QI to verify the aerosol concentrations determined by weighing the filters and to ascertain the purity of the aerosol during the exposure. These results, shown in Table 5, demonstrate that the characteristics of the dye aerosol were stable during the study and concentrations based on the mass of the filter samples were accurate. The variation in the data presented in Table 5 can be attributed to experimental errors during these measurements, since no statistical differences were found.

Clinical Observations

Clinical observations were performed on all animals before exposures to ensure that they were in good health. Clinical observations were also done at 2 weeks after the initiation of exposures and at the end of the 4-week exposures. In all observations, no adverse health conditions were noticed in any of the animals.

Animal Body Weights

Table 6 summarizes the average weights of the male and female rats at the end of the 4-week repeated inhalation exposure to SY. Before exposure, there was no statistical difference in the weights of the animals between the various exposure groups. However, after the 4-week exposure period, animals exposed to the highest concentration of SY dye aerosol (230 mg/m^3) weighed 8 percent less than the control animals (for the null hypothesis $p < 0.01$ in a multiple comparison t-test).

TABLE 5

Analysis of SY Dye Aerosols for QI* During the 4-Week Exposure

<u>Week</u>	<u>QI Content</u> <u>(mass QI/mass dye)</u>
1	0.90 \pm 0.02
2	0.97 \pm 0.07
3	0.95 \pm 0.10
4	0.92 \pm 0.01

Values are mean \pm SE. n = 3. No statistical differences were seen between the four groups in multiple comparison t-tests of the means (for the null hypothesis $p > 0.2$).

*QI = 2-(2'-quinolyl)-1,3-indandione, the major component of yellow dye

TABLE 6

Average Weights of Male and Female Rats Before and After
Repeated Inhalation Exposure to SY for 4 Weeks^a

		<u>Exposure Chamber</u>							
		<u>Control</u>		<u>Low</u>		<u>Medium</u>		<u>High</u>	
		<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>
Before									
Exposure		276±3	166±2	269±5	168±2	273±4	167±2	274±3	166±3
After									
Exposure		298±2	176±2	292±4	181±2	292±3	178±2	269±3 ^b	166±1 ^b
		n = 30		n = 20		n = 20		n = 30	

^a Average animal weight (grams) ± SE.

^b Statistically different from controls in multiple comparison t-tests
(for the null hypothesis $p < 0.01$).

Lung Content of QI After Exposure to SY Dye

On the day after the last exposure day, lungs from six rats (three male and three female) from each chamber (low, medium and high concentration) were analyzed for QI content. Animals were sacrificed and lungs removed, with care to avoid contamination with yellow dye on the pelt. Each lung was homogenized in 1.5 mL of acetonitrile and centrifuged, and the supernatant was removed. The pellet was resuspended in 2 mL acetonitrile, centrifuged, and the supernatant removed. This procedure was repeated three more times. Preliminary experiments in which excised control lung lobes were directly injected with a syringe with a known amount of SY dye dissolved in acetonitrile prior to homogenization showed that this extraction procedure removed 100 percent of the QI. The combined supernatants were diluted to 10 mL and analyzed for QI by HPLC using the following conditions:

reverse phase column (Alltech C-18, 10 μ ; 25 cm x 4.6 mm)
9:1 acetonitrile:water mobile phase (1 mL/min)
UV detection at 435 nm

The results, shown in Table 7, demonstrate that very little QI was retained in the lung. Thus, the majority of the QI deposited in lungs during exposures was cleared at a rapid rate. Assuming 10 percent pulmonary deposition of inhaled SY dye aerosol⁷ and a minute volume of 200 mL/min, 1.8 mg of SY dye/day should have been deposited in animals exposed to the high concentration. Of the amount calculated to be deposited during only the last day of exposure, only ~ 0.2 percent remained 16 hours after exposure.

Respiratory Function Measurements

The respiratory function of 16 control rats and 16 treated rats exposed to the highest concentration of SY (230 mg/m³) was measured. The rats were anesthetized with halothane and tested by plethysmography using methods

TABLE 7

Lung Concentrations of QI* Following a 4-Week
Repeated Inhalation Exposure to SY Dye Aerosol

<u>Exposure Concentration</u> <u>(mg/m³ ± SD; n = 22)</u>	<u>Sex</u>	<u>QI Concentration in Lung</u> <u>(ng/g lung ± SE; n = 3)</u>
10 ± 5	M	210 ± 80
	F	230 ± 30
51 ± 10	M	790 ± 200
	F	1300 ± 1000
230 ± 30	M	3500 ± 1700
	F	2300 ± 600

*QI = 2-(2'-quinoly1)-1,3-indandione.

previously reported.⁸ Group mean values were calculated for before-exposure and after-exposure data. The significances of differences between mean values for control and treated rats were estimated by a two-tailed Student's t-test.

A portion of the respiratory function data are presented in Table 8. Both dynamic and quasistatic lung compliance were greater in treated than in control rats after the exposure. Quasistatic compliance (both chord and maximum) was slightly greater in treated than in control rats before exposure, and the difference was significant after exposure. Total lung capacity was not different before or after exposure; however, the total lung capacity of control rats increased 0.7 mL during the 5 weeks and that of treated rats increased only 0.4 mL. Because of the body weight difference, the total lung capacity/kg body weight of treated rats after exposure was higher than that of control rats. The functional residual capacities of the two groups were similar before exposure, but the functional residual capacity of the treated group was larger than that of the control group after exposure. There was a trend toward a higher functional residual capacity/total lung capacity ratio in the treated group after exposure. The forced vital capacity of treated rats was larger than that of control rats after exposure. The fraction of forced vital capacity expired in 0.1 sec increased during the 4 weeks in control rats, but decreased slightly in treated rats, although the difference after exposure was not statistically significant. Similarly, the peak expiratory flowrates were not significantly different between the two groups before or after exposure, but this parameter increased in control rats during the 5 weeks while it did not in treated rats. None of the absolute forced expiratory flowrates were significantly different between groups after exposure. However, since the forced vital capacity of treated rats was

TABLE 8

Respiratory Function of Control and Treated (230 mg/m³) Rats Before and After a 4-Week Repeated Inhalation Exposure to St Hye (n = 16 each group)

Parameter	Units	Before Exposure			After Exposure		
		Control	SE	High Dose	Control	SE	High Dose
		\bar{x}	\pm	\bar{x}	\bar{x}	\pm	\bar{x}
Body Weight	g	208	13	215	236	15	216
Dynamic Lung Compliance	ml/cm H ₂ O	0.34	0.02	0.33	0.29	0.01	0.39*
Quasistatic Chord Compliance	ml/cm H ₂ O	0.68	0.03	0.72	0.66	0.04	0.77*
Maximum Quasistatic Compliance	ml/cm H ₂ O	0.93	0.05	1.11*	0.85	0.05	1.10*
Total Lung Capacity (TLC)	ml	11.8	0.4	12.4	12.2	0.5	13.1
TLC/kg Body Wt	ml/kg	58	2	60	53	2	63*
Functional Residual Capacity (FRC)	ml	2.7	0.1	2.8	2.7	0.1	3.2*
FRC/TLC		0.23	0.01	0.22	0.23	0.01	0.26
Forced Vital Capacity	ml	10.1	0.4	10.7	10.0	0.4	11.5*
% FVC Exhaled in 0.1 Sec	%	69	1	67	71	2	56
Peak Expiratory Flowrate (PEFR)	ml/sec	109	3	111	112	3	111
PEFR/FVC	ml/sec/ml	10.9	0.2	10.5	11.2	0.2	9.8*
Mean Widespiratory Flowrate (MREF)	ml/sec	69	1	70	74	2	75
MREF/FVC	ml/sec/ml	6.9	0.2	6.6	7.5	0.3	6.6*
Expiratory Flowrate at 50% FVC (EF ₅₀)	ml/sec	71	2	83	77	3	79
EF ₅₀ /FVC	ml/sec/ml	7.1	0.2	6.9	7.8	0.3	6.9*
Expiratory Flowrate at 25% FVC (EF ₂₅)	ml/sec	45	1	45	46	2	47
EF ₂₅ /FVC	ml/sec/ml	4.3	0.2	4.2	4.7	0.2	4.1*
Expiratory Flowrate at 10% FVC (EF ₁₀)	ml/sec	21	1	22	21	1	21
EF ₁₀ /FVC	ml/sec/ml	2.1	0.1	2.0	2.2	0.1	1.8*
CO Diffusing Capacity	ml/min/mmHg	0.182	0.011	0.196	0.196	0.010	0.208
DLCO/kg	ml/min/mmHg/kg	0.880	0.023	0.927	0.848	0.022	0.984*
DLCO/ml Alveolar Volume	ml/min/mmHg/ml	0.0169	0.0006	0.0176	0.0178	0.0006	0.0168

*Statistically different from controls by the Student's t-test of the means (for the null hypothesis $p < 0.05$).

greater, the volume-normalized flowrates (flows/forced vital capacity) were significantly lower in treated rats. The CO diffusing capacity of treated rats was slightly higher than that of control rats both before and after exposure, and the CO diffusing capacity of both groups increased during the 5 weeks between tests. Because of the weight difference, the CO diffusing capacity/kg of treated rats was higher than that of control rats after exposure. The volume-normalized CO diffusing capacity was slightly lower in treated rats after exposure.

The changes caused by dye exposure can be summarized as a decreased lung elastic recoil, and increased resting lung volume and slight forced airflow obstruction. This pattern of differences was consistent with mild emphysematous changes in the lung. The degree of abnormality observed in treated rats was slight compared to that seen in established emphysema induced experimentally in rats.⁸ Emphysema is usually a chronic, progressive process. It can be expected that if this high concentration of inhaled solvent yellow dye does result in morphologic changes of emphysema in rats, a longer exposure might be needed to see the effect histologically.

Lung Biochemistry

Lung damage following the 4-week SY inhalation exposure was evaluated by analysis of bronchoalveolar lavage (BAL) fluid. Previous studies have shown this to be a useful method to detect an inflammatory response in lungs following inhalation of toxic materials.⁹ Results from these analyses did not show any dose-related statistically significant changes in any of the exposure groups as compared to controls (Table 9). There was a slight increase in the number of neutrophils in BAL from exposed animals, but the increases were not dose-related. Only the BAL from control and high level

TABLE 9

Bronchoalveolar Lavage Fluid Analysis Following
Inhalation Exposures of F344 Rats to SY Dye(X \pm SE, n = 12)

Parameter ^a	Exposure Concentration			
	Control	Low	Medium	High
Lactate Dehydrogenase, mIU/g	660 \pm 50	640 \pm 60	600 \pm 40	627 \pm 50
β -Glucuronidase, mIU/g	1.5 \pm 0.3	1.2 \pm 0.2	1.4 \pm 0.1	1.2 \pm 0.2
Acid Phosphatase, mIU/g	23 \pm 1	17 \pm 1	16 \pm 2	18 \pm 2
Alkaline Phosphatase, mIU/g	400 \pm 20 ^c	300 \pm 20	260 \pm 20	260 \pm 20
Glutathione Reductase, mIU/g	48 \pm 5	54 \pm 6	50 \pm 4	74 \pm 9
Acid Proteinase, mg/hr/g ^b	0.7 \pm 0.2	not done	not done	1.1 \pm 0.3
Cathepsin D	0.7 \pm 0.2	not done	not done	0.5 \pm 0.1
Cathepsin B	0.0 \pm 0.3	not done	not done	0.6 \pm 0.3
Protein, mg/g	4.3 \pm 0.6	2.2 \pm 0.4	1.7 \pm 0.2	1.7 \pm 0.2
Macrophages, 10 ³ cells/g	1200 \pm 160	1700 \pm 380	1500 \pm 260	2000 \pm 500
Neutrophils, 10 ³ cells/g	0	100 \pm 80	16 \pm 5	100 \pm 12

^aValues are normalized to lu. wet weight. Mean lung weight was not statistically different between groups.

^bActivity is reported as mg protein digested per hr per g lung. See Table 10 for definition of Cathepsin D and E activities.

^cStatistically different from the exposed groups by multiple comparison t-tests of the means (for the null hypothesis $p < 0.05$).

exposed rats were analyzed for proteinase activity to avoid analyzing all samples if no response was seen at the highest exposure level. The slightly elevated alkaline phosphatase in BAL from control animals is unexplained. Historical control values from this laboratory indicate a 95 percent confidence interval for control values of 231 to 265 mIU/g lung for this parameter (range of individual values = 95-480 mIU/g lung).

Lung tissue proteolytic activity was also measured in the control and high level exposed rats (Table 10). Increases in tissue acid proteinase activity were observed which is consistent with an influx of inflammatory cells or an "activation" of cells already present. The major increase in acid proteinase activity was associated with the activity inhibited by leupeptin (i.e., "cathepsin B-like" activity).

Lung Connective Tissue Biochemistry

Total lung and airway fluid hydroxyproline content was measured in six control rats and six rats exposed to the highest level (230 mg/m³) of SY for 4 weeks. In the lung tissue, hydroxyproline content can be converted to collagen content, because hydroxyproline represents 13 percent of collagen by weight. This was expressed as mg collagen/gram control lung so that increases in wet weight of exposed lungs from injury or inflammation processes would not mask any increase in lung collagen. No increase or decrease in total lung collagen content was seen in SY-exposed animals as compared to control rats, as judged by a two-tailed Mann-Whitney U statistical test at a significance level of $p \leq 0.05$ (Table 11).

Hematology and Serum Clinical Chemistries

Blood was obtained by cardiac puncture following halothane anesthesia, and cervical dislocation of six male and six female rats at each exposure

TABLE 10

Lung Proteinolytic Activity in Rats After a
4-Week Repeated Inhalation Exposure to SY Dye^a

	<u>Control</u>	<u>High</u>
<u>Lung Tissue:</u>		
Total Acid Proteinase	18 ± 2	32 ± 3 ^f
Cathepsin D ^b	12 ± 1	16 ± 1 ^f
Cathepsin B ^c	6 ± 3	16 ± 2 ^f
Total Neutral Proteinase	0.7 ± 0.1	1.3 ± 0.5
Macrophage Elastase ^d	0.0 ± 0.2	0.7 ± 0.4
Cathepsin G +	0.8 ± 0.2	0.6 ± 0.1
Neutrophil Elastase ^e		

^aMean ± SE (n = 6) proteinase activity as mg protein released per hour and normalized to the average lung weight from control animals.

^bNot inhibited by Leupeptin.

^cInhibited by Leupeptin.

^dInhibited by 1,10 phenanthroline.

^eNot inhibited by 1,10 phenanthroline.

^fSignificantly different from controls by the Mann-Whitney U statistic (for the null hypothesis p < 0.05).

TABLE 11

Total Lung Collagen Levels in Rats Exposed to
SY Dye Aerosol for Four Weeks

	<u>Total Collagen Content</u>	
	<u>Lung Collagen</u> <u>(mg/g control lung)^c</u>	<u>Lavage Fluid Peptides^b</u> <u>(mg/g control lung)^c</u>
Control	8.3 ± 0.4	56 ± 14
SY Exposed (High Level)	9.1 ± 0.7	44 ± 8

^aMean ± SE (n = 6).

^bDetermined by analysis of bronchoalveolar lavage fluid.

^cThe amount of lung collagen or lavage fluid peptides normalized to the average lung weight from control animals. No differences were found using the Mann-Whitney U statistical test ($p > 0.05$ for the null hypothesis in all comparisons).

level after completion of the 4 week exposure. One mL of whole blood in anticoagulant (oxalate) from each rat was prepared for hematology. An additional 5 mL of blood was processed for serum clinical chemistry measurements by centrifugation at 1000 x G for 20 minutes and the non-hemolyzed supernatant serum analyzed. Table 12 lists the hematology and serum clinical chemistry variables that were measured. The values obtained from the three levels of dye exposure were compared to those of the control group. Serum electrolyte analysis showed that the total CO_2 in rat sera was statistically different from controls in all three levels of exposure. Inorganic phosphorus, cholesterol, and glucose were also modestly increased in an exposure-level related manner (Table 13). However, the importance of these small increases is unknown. No other exposure related differences in blood chemistry parameters were noted between exposed and control rats (Table 13).

Immunology (Skin Sensitivity)

SY is a known allergic sensitizer in humans.¹⁰ Therefore, experiments were conducted to evaluate if hypersensitive skin reactions occurred from repeated SY exposures. Groups of eight control and eight high level exposed rats (equally divided by sex) were injected intradermally in the ear with either saline vehicle or the SY dye. In 4 rats from each group, injections were 500 μg of SY, a dose which does not cause irritation. The remaining 4 rats in each group were injected with 0.1 mL saline. Injections were completed within 24 hours after the end of the 4-week exposures. The reactions in the ears injected with dye and saline were evaluated at 15-30 minutes, 8, 24, and 48 hours after injection for the evaluation of Type I (IgE mediated), Type III (Arthus), and Type IV hypersensitivity. Results indicated

TABLE 12

Hematology and Serum Clinical Chemistry Measurements on Rats
Exposed by Inhalation to SY or SY/SG Dye Aerosols for Four Weeks

a. Hematology:

- 1) Hematocrit
- 2) Hemoglobin concentration
- 3) Erythrocyte count
- 4) Erythrocyte indices (mean cellular volume, mean cellular hemoglobin, mean cellular hemoglobin concentration)
- 5) Leukocyte count, total
- 6) Leukocyte count, differential

b. Serum Chemistry:

- 1) Total protein and albumin
- 2) Serum gamma-glutamyl transpeptidase
- 3) Serum glutamic pyruvic transaminase
- 4) Serum alkaline phosphatase
- 5) Bilirubin (total)
- 6) Blood urea nitrogen
- 7) Creatinine
- 8) Calcium
- 9) Phosphorus
- 10) Sodium
- 11) Potassium
- 12) Chloride
- 13) Glucose
- 14) Cholesterol
- 15) Thyroxine (T_4)

TABLE 13

Changes in Selected Clinical Chemistry Measurements
After Inhalation of SY DyeMean \pm SE (n = 11-12)

Parameter	Control	Exposure Concentration		
		Low	Medium	High
Total CO ₂ (mMoles/L)	18 \pm 1	20 \pm 1*	20 \pm 1*	21 \pm 1*
Alkaline Phosphatase (IU/L)	90 \pm 1	78 \pm 2*	100 \pm 2*	93 \pm 1*
Total Bilirubin (mg/dL)	0.8 \pm 0.2	0.7 \pm 0.1	0.5 \pm 0.1	0.6 \pm 0.1
Total Protein (g/dL)	6.4 \pm 0.2	6.5 \pm 0.1	6.3 \pm 0.1	6.4 \pm 0.1
Albumin (g/dL)	4.4 \pm 0.5	4.9 \pm 0.2	4.2 \pm 0.2	3.9 \pm 0.5
Albumin/Globulin	1.8 \pm 0.3	2.5 \pm 0.2	2.2 \pm 0.4	2.5 \pm 0.6
BUW (mg/dL)	23 \pm 1	26 \pm 1	26 \pm 2	24 \pm 5
Creatinine (mg/dL)	0.6 \pm 0.1	0.6 \pm 0.1	0.6 \pm 0.1	0.6 \pm 0.1
Inorganic Phosphorus (mg/dL)	5.5 \pm 0.2	7.5 \pm 0.3*	8.6 \pm 0.5*	8.4 \pm 0.2*
Cholesterol	71 \pm 6	92 \pm 6*	126 \pm 11*	110 \pm 5*
Glucose	190 \pm 10	231 \pm 14*	293 \pm 26*	277 \pm 15*

*Statistically different from control according to multiple comparison t-tests of the means
(for the null hypothesis $p < 0.05$).

no hypersensitivity reactions to have occurred in exposed rats as compared to controls. However, it must be noted that the rat is not an ideal animal model for studying skin sensitivities.

Histopathology

Histopathological evaluations of tissues taken from control, medium and high level exposure rats were done at Experimental Pathology Laboratories, Inc. (Herndon, VA). Routine hematoxylin and eosin stained slides were prepared from the lung (4), larynx, trachea, nasal cavity (4), skin, tracheobronchial lymph node, popliteal lymph node, spleen, femur, heart, stomach, duodenum, cecum, colon, liver, pancreas, kidney, urinary bladder, epididymis, testis, prostate, uterus, ovary, adrenal, thyroid, brain, pituitary, and eye for a total of thirty-two tissues for males and thirty-one for females. These slides were examined microscopically, and histopathologic evaluations were made for each animal.

Examination of tissues from ten male and ten female rats exposed by inhalation to either the medium or high dose of SY dye failed to reveal any exposure-related effect when the findings were compared to those from a similar set of male and female control rats. Most of the rats including the controls had minimal to slight proliferations of lymphoid cells in pulmonary perivascular and peribronchiolar areas. No lesions were observed in the larynx, trachea, or the anterior region (Level I) of the nasal cavity of any of the rats. There were minimal to slight lymphoid infiltrates under the epithelium lining the dorsal meatus or around the nasolacrimal duct in some of the other levels of the nasal cavities in all groups including the controls. This finding was more common in the posterior region (Level III) of the nasal cavity of both sexes. Other changes appeared to be spontaneous lesions or

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incidental findings for this group of rats, for example, thyroglossal thyroid cysts, pituitary cysts, and intestinal nematodiasis. Therefore, it was concluded that no exposure-related lesions were observed in tissues from rats exposed to the medium or high dose of SY dye.

FOUR-WEEK INHALATION EXPOSURE TO SY/SG DYE

Nominal exposure concentrations selected for this study were 0, 10, 50 and 250 mg/m³ given 6 hours/day, 5 days/week, for 4 weeks. These exposure concentrations were selected on the basis of the same criteria as the 4-week SY dye exposure previously mentioned.

Statistical Analysis

Where possible, computer software packages such as BMDP or RSI were used for data handling and analysis. Standard tests applied to the experimental data were one-way analyses of variance, followed by tests for the equality of means, such as simple t-tests, multiple comparison t-tests,⁴ or non-parametric Mann-Whitney U tests. Usually a confidence level of 0.05 was used, although in some cases significance was expressed in much smaller or much larger values.

Animals and Animal Exposures

Specific pathogen free, male and female Fischer-344 (F344/Crl Lov) rats, 15-20 weeks of age with an age range of ± 2 weeks, were used for this study. These animals were obtained from an existing colony raised at the Inhalation Toxicology Research Institute. Rats were then acclimatized in exposure chambers, randomized into experimental groups, and cared for similarly to that described previously in this report for the four-week inhalation exposure study of SY dye.

Animals were exposed to 3 graded levels of SY/SG dye material generated by a Jet-O-Mizer air jet mill as previously described.^{5,6} The arrangement and entry groups of animals in the four Hazleton 2000 whole-body inhalation chambers used for this study (3 exposure and 1 air control), and chamber environment monitoring and maintenance were as previously described in this

report for the four-week inhalation study to SY dye. Concentration and size characteristics of the exposure atmospheres for each of the 22 exposure days are shown in Table 14. The SY/SG aerosol size was measured with a Lovelace Multi-Jet cascade impactor each day during the first week of exposure and at intervals as shown in Table 14 thereafter. Table 15 shows the average SY/SG concentration, while Table 16 shows the average mass median aerodynamic diameter and average geometric standard deviation of these aerosols with respect to the different entry groups of animals at each exposure concentration. No statistical differences were seen between the three entry groups for each of the exposure concentrations in each case. The systematic trend to larger particle size with higher dye exposure concentration is again seen. But this time the differences between the diameters of the high and low exposure concentration groups are significant ($p < 0.05$ for the null hypothesis). However, the effect of these small differences in particle size on the fractional respiratory tract deposition of the inhaled SY/SG dye aerosol would be minimal.⁷

Clinical Observations

Clinical observations were performed on all animals before exposures to ensure that they were in good health. Clinical observations were also done at 2 weeks after the initiation of exposures and at the end of the 4-week exposures. In all observations, no adverse health conditions were noticed in any of the animals.

Animal Body Weights

As with the rats exposed for four weeks to SY, SY/SG exposed animals showed weight differences after exposure as compared to controls, which were not present before exposure. Table 17 shows that both male and female rats

TABLE 14

Daily Exposure Chamber Concentration and
Particle Size of SY/SG Dye Aerosols

	Chamber 1			Chamber 2			Chamber 3		
	Conc ₃ (mg/m ³)	MMAD ^a (μm)	σ _g ^b	Conc ₃ (mg/m ³)	MMAD (μm)	σ _g	Conc ₃ (mg/m ³)	MMAD (μm)	σ _g
Day 1	9.9	3.6	2.0	53.5	4.1	2.1	191	4.4	1.8
2	14.0	3.8	2.3	54.5	4.1	2.0	180	4.8	1.9
3	29.9	3.2	2.0	39.3	4.2	2.0	208	5.4	2.0
4	9.9	3.0	1.9	42.8	3.0	1.8	305	5.3	2.0
5	10.5	2.9	1.9	39.1	2.9	1.8	257	5.0	1.9
6	12.4	---	---	24.6	---	---	265	---	---
7	6.9	---	---	34.6	---	---	192	4.3	1.8
8	8.6	3.0	1.9	39.5	---	---	186	---	---
9	10.1	---	---	65.0	---	---	236	---	---
10	9.7	---	---	60.5	3.8	1.9	199	---	---
11	11.3	---	---	62.7	---	---	180	---	---
12	10.2	2.8	1.9	42.5	3.9	2.0	182	---	---
13	8.2	---	---	38.1	---	---	181	4.1	2.0
14	11.1	---	---	56.2	---	---	129	---	---
15	11.2	---	---	40.0	---	---	172	---	---
16	10.0	---	---	43.8	---	---	188	5.6	2.0
17	10.8	---	---	43.8	---	---	332	---	---
18	8.8	3.1	2.0	55.1	3.9	1.9	159	---	---
19	10.4	---	---	62.6	---	---	206	---	---
20	9.8	---	---	64.6	---	---	144	---	---
21	10.7	---	---	61.6	---	---	263	---	---
22	5.8	---	---	45.8	---	---	263	---	---
\bar{X}	11	3.2	2.0	49	3.7	1.9	210	4.9	1.9
S. D.	±5	0.4	0.1	±11	0.5	0.1	±50	0.6	0.1

Daily Chamber concentrations were determined by the mean of 6 to 8 filter samples taken at regular intervals during the day. The average variance among the daily filter samples collected from the low, medium and high dose chambers was 21 percent, 19 percent and 22 percent, respectively.

^aMMAD = mass median aerodynamic diameter.

^bσ_g = geometric standard deviation.

TABLE 15

Average SY/SG Aerosol Concentrations Measured During the
4-Week Repeated Inhalation Exposure of Rats*

	<u>Exposure Chamber</u>		
	<u>Low</u>	<u>Medium</u>	<u>High</u>
Group I	11.2 ± 1.0	48 ± 3	205 ± 11
Group II	11.2 ± 1.0	49 ± 3	208 ± 12
Group III	10.8 ± 1.0	48 ± 3	212 ± 12

*Average Concentration of SY/SG (mg/m^3) ± SE (n = 20). Values were not found to be statistically different between the three animal entry group at each exposure concentration using multiple comparison t-tests of the means (for the null hypothesis $p > 0.1$).

TABLE 16
Average Particle Size of SY/SG Aerosols Used in
the 4-Week Repeated Exposure of Rats

	<u>SY/SG Exposure Concentrations</u>		
	<u>Low</u>	<u>Medium</u>	<u>High</u>
Group I*			
MMAD**	3.2 ± 0.4	3.7 ± 0.5	4.9 ± 0.6
σ_g ***	2.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
Group II*			
MMAD	3.1 ± 0.3	3.7 ± 0.5	4.9 ± 0.6
σ_g	2.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
Group III*			
MMAD	3.0 ± 0.1	3.6 ± 0.5	4.9 ± 0.6
σ_g	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1

*Multiple comparison t-tests between the 3 group means show no differences of significance (for the null hypothesis $p > 0.2$). However, there is a systematic trend to larger particle diameters with higher SY/SG exposure concentration groups. The differences between the high and low concentration exposure groups are significant (for the null hypothesis $p < 0.05$).

**Average Mass Median Aerodynamic Diameter ± SD (n = 8).

***Average Geometric Standard Deviation ± SD (n = 8).

TABLE 17

Average Weights of Male and Female Rats Before and After
Repeated Inhalation Exposure to SY/SG for 4 Weeks^a

	<u>Exposure Chamber</u>							
	<u>Control</u>		<u>Low</u>		<u>Medium</u>		<u>High</u>	
	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>
Before								
Exposure	266±3	163±2	270±5	159±2	270±4	157±4	267±3	160±2
After								
Exposure	289±5	176±3	296±6	174±2	295±4	172±2	270±3 ^b	163±1 ^b
	n = 34		n = 23		n = 23		n = 34	

^aAverage animal weight (grams ± SE).

^bStatistically different from controls using multiple comparison t-tests
of the means (for the null hypothesis $p < 0.05$).

exposed to the highest level of SY/SG gained weight at a significantly slower rate as compared to the other animal exposure groups including controls.

Lung Content of QI and TA After Exposure to SY/SG Dye

Lungs from 3 male and 3 female rats from each chamber were analyzed for the two components of green dye (QI and TA) by HPLC using the following conditions:

reverse phase column (Alltech C-18, 10 μ ; 25 cm x 4.6 mm)

95.5 acetonitrile: water mobile phase, 1 mL/min

UV detection at 435 and 620 nm

Lungs were homogenized in acetonitrile, centrifuged, and the supernatant removed. The pellet was repeatedly extracted with more solvent until no more color was observable in the solvent. Little QI was present in any group. There appeared to be little difference between male and female rats in the amount of TA retained. The amount of TA in the lungs increased with increasing aerosol concentration, but not in a linear relationship (Table 18).

A significant amount (14-33 percent of the total TA that was inhaled was still present in the lung at the end of the exposure. Table 19 compares the amount of TA in the lung at sacrifice with the calculated amount deposited during the 20 days of exposure. This is an important observation because inhaled organic compounds are rarely retained in the lung, and studies have shown that increased lung retention correlates with increased toxic effects in the lung for certain compounds.

Respiratory Function Measurements

Sixteen control and 16 rats exposed to the highest level of SY/SG dye (210 mg/m³) were evaluated by respiratory function tests before and after the 4-week inhalation exposure as described previously for the SY exposures.

TABLE 18

Lung Content of QI* and TA** After the Four-Week
Inhalation Exposure to SY/SG Dye

Aerosol Concentration (mg/m ³)	Lung Concentration (μ g/g Lung \pm SE, n = 3)	
	QI	TA
<u>Male</u>		
Control	< 0.1	1.0 \pm 0.2
11	< 1	290 \pm 20
49	< 5	740 \pm 60
210	< 10	2110 \pm 240
<u>Female</u>		
Control	< 0.1	0.9 \pm 0.3
11	< 1	250 \pm 30
49	< 5	650 \pm 90
210	< 10	2150 \pm 80

*2-(2'quinoly1)-1,3-indandione

**1,4-di-p-toluidinoanthraquinone

TABLE 19

Lung Deposition and Retention of TA

<u>Aerosol Concentration ($\mu\text{g/L}$)</u>	<u>Amount Retained Mean \pm SE (mg/Lung)</u>	<u>Calculated Amount Deposited^a (mg/Lung)</u>	<u>Fraction (%) Retained^b</u>
Male			
11	0.35 \pm 0.04	1.0	33
49	0.89 \pm 0.07	4.7	19
210	2.97 \pm 0.30	20	15
Female			
11	0.24 \pm 0.03	0.8	30
49	0.58 \pm 0.09	3.5	16
210	2.08 \pm 0.18	15	14

^a Assuming 20 exposures at 6 hr per exposure, 200 mL minute volume for males and 150 mL for females, 10 percent lung and bronchial deposition, and 67 percent of SY/SG is TA.

^b The fraction retained compares the amount of TA in the lung at sacrifice with the calculated amount deposited during the 20 days of exposure.

TABLE 20

Respiratory Function of Rats Before and After
4-Week Exposure to SY/56 Bye (210 mg/m³)
(n = 16 per group)

Parameter	Units	Baseline			Post-Exposure		
		Control $\bar{x} \pm$ SE	High Dose $\bar{x} \pm$ SE		Control $\bar{x} \pm$ SE	High Dose $\bar{x} \pm$ SE	
Body weight	g	203	214	12	226	211	12
Quasistatic chord compliance	mL/cmH ₂ O	0.63	0.67	0.02	0.69	0.68	0.04
Total lung capacity (TLC)	mL	12.6	12.7	0.3	13.1	12.6	0.6
Vital capacity (VC)	mL	9.7	10.0	0.4	10.6	10.7	0.5
VC/TLC		0.77	0.79	0.01	0.80	0.84*	0.01
Functional residual capacity	mL	3.4	3.3	0.2	3.6	3.2	0.2
FRC/TLC		0.27	0.26	0.01	0.27	0.25	0.01
Residual Volume (RV)	mL	2.8	2.6	0.2	2.5	2.1*	0.1
RV/TLC		0.23	0.21*	0.01	0.19	0.16*	0.01
CO diffusing capacity (DLCO)	mL/min/mmHg	0.19	0.20	0.01	0.20	0.17	0.01
DLCO/kg body wt.	DLCO/g	0.89	0.94	0.03	0.92	0.84*	0.03
DLCO/mL alveolar volume	DLCO/mL	0.018	0.018	0.001	0.018	0.015*	0.001
Forced vital capacity (FVC)	mL	9.9	10.3	0.3	10.9	10.9	0.6
% FVC expired in 0.1 sec.	%	66.8	67.9	1.4	64.8	62.5	2.0
Mean midexpiratory flowrate (MMEF)	mL/sec	71.2	75.3	2.2	75.8	67.2*	2.7
MMEF/FVC	mL/sec/mL	7.2	7.4	0.2	7.0	6.3	0.4
Expiratory flow at 50% FVC (EF ₅₀)	mL/sec	73.7	80.3	2.5	79.4	69.0*	5.4
EF ₅₀ /FVC	mL/sec/mL	7.5	7.8	0.2	7.2	6.5	0.4
Expiratory flow at 25% FVC (EF ₂₅)	mL/sec	45.8	47.4	2.0	50.1	42.9*	1.8
EF ₂₅ /FVC	mL/sec/mL	4.6	4.7	0.1	4.6	4.1	0.2
Expiratory flow at 10% FVC (EF ₁₀)	mL/sec	21.4	22.6	0.9	24.1	22.1	1.1
EF ₁₀ /FVC	mL/sec/mL	2.2	2.2	0.1	2.1	2.1	0.1

*Statistically different from controls by the Student's t-test of the means (for the null hypothesis $p < 0.05$).

The significance of differences between control and exposed rat mean values were estimated by a two-tailed Student's t-test. A portion of the summary data are presented in Table 20, along with results of the statistical tests.

The total lung capacity, vital capacity, and functional residual capacity of the control rats increased during the exposure period more than those of exposed rats. This effect is consistent with a retardation of normal growth in the exposed rats. The residual volume and residual volume/total lung capacity of the treated rats were significantly lower than those of controls after exposure. A reduction in alveolar-capillary gas exchange ability of the exposed rats was reflected by their lower values for diffusing capacity expressed per gram body weight and per mL lung volume. The forced expiratory flowrates of the exposed rats were significantly lower than those of the control rats at the end of the exposure. Volume-normalized flowrates were also lower for the treated group, but not significantly.

In brief, the dye exposure caused a trend toward smaller lung volumes, reduction of gas exchange efficiency, and slight airflow obstruction in rats exposed to the highest concentration of dye.

Lung Biochemistry

Bronchoalveolar lavage fluid (BAL) from the rats exposed to SY/SG dye mixture indicated an inflammatory response in rats exposed to the highest concentration (Table 21). The elevation in cytoplasmic enzymes in BAL (lactate dehydrogenase (LDH), glutathione reductase, and glutathione peroxidase) indicates the SY/SG dye mixture caused some cell damage. The increased activity of the lysosomal enzyme, β -glucuronidase, is consistent with what has been seen when other insoluble particles (quartz, fly ash, diesel soot) are deposited in the lung.¹¹⁻¹³ This enzyme is released from

TABLE 21

Changes in Bronchoalveolar Lavage Fluid in Response to Inhaled SY/S6 Dye Mixture

Parameter ^a	Exposure Concentration			
	Control	Low	Medium	High
Lactate Dehydrogenase, mIU/g	450 ± 40	460 ± 20	530 ± 50	1730 ± 80*
β-Glucuronidase, mIU/g	1.3 ± 0.2	1.2 ± 0.3	1.7 ± 0.2	12.1 ± 0.8*
Acid Phosphatase, mIU/g	19 ± 1	18 ± 1	16 ± 1	21 ± 1
Alkaline Phosphatase, mIU/g	160 ± 12 ^c	270 ± 15	260 ± 17	295 ± 15
Glutathione Reductase, mIU/g	49 ± 6	50 ± 3	52 ± 3	99 ± 6*
Glutathione Peroxidase, mIU/g	9.3 ± 1.6	9.3 ± 0.7	9.1 ± 0.8	15.0 ± 1.3*
Acid Proteinase, mg/nr/g ^b	0.34 ± 0.05	0.25 ± 0.03	0.17 ± 0.03	1.85 ± 0.23*
Cathepsin D	0.13 ± 0.05	0.13 ± 0.02	0.16 ± 0.02	1.33 ± 0.14*
Cathepsin B	0.21 ± 0.10	0.13 ± 0.02	0.10 ± 0.04	0.55 ± 0.24
Protein, mg/g	1.4 ± 0.1	1.6 ± 0.1	2.0 ± 0.1*	3.6 ± 0.2*
Macrophages, 10 ³ cells/g	450 ± 80	840 ± 100*	570 ± 60	1080 ± 150*
Neutrophils, 10 ³ cells/g	2 ± 1	33 ± 6*	290 ± 94*	1720 ± 240*

*Value differs from control value in a multiple comparison t-test of the means ($p < 0.05$ for the null hypothesis).

^aValues are normalized to lung wet weight. Mean lung weights did not vary between groups.

^bActivity is reported as mg of protein digested per hr per g lung.

^cLow value, significantly different from the exposed groups in multiple comparison t-tests of the means ($p < 0.05$ for the null hypothesis).

both macrophages and neutrophils upon appropriate stimulation.¹³⁻¹⁵ Acid phosphatase, another lysosomal enzyme, is not elevated and this is also consistent with what has been observed in rats exposed to other insoluble particles. Alkaline phosphatase activity in the BAL from control rats was lower than our historical controls (see page 26) for unknown reasons. No dose-related response was observed for this parameter.

There was increased acid proteinase activity in BAL. Again, changes were noted only at the highest exposure level. The increase was mostly resistant to inhibition by leupeptin, suggesting it to be Cathepsin D. This increase suggested that an inflammatory process was in progress in lungs of rats which inhaled green munition dyes. The more modest increase in Cathepsin B suggested that the turnover of pulmonary architecture was less important than the cleanup of cellular or particle debris which is indicated by an increase in Cathepsin D.

The neutrophil cell counts increased in a dose dependent fashion in the exposed rats. Macrophage counts were approximately doubled, but only in the high level exposed animal. The combined increase in both cell types and in protein content of BAL indicates an inflammatory response in the high level exposed rats. The increase in neutrophils in medium level exposed rats indicates a mild inflammatory response. These results are consistent with the histopathology findings reported below.

Lung tissue of rats exposed to the highest level of SY/SQ for four weeks were also analyzed and showed increased acid proteinase activities (Table 22) relative to those of controls. Comparisons were made between the means of each of the three exposure groups and of the control group using the Mann-Whitney U statistical test. Inhibitor profiles indicated that most of

TABLE 22

Lung Tissue Proteinase Activities of Rats
Exposed to SY/SG Dye for 4 Weeks^a

	Acid Proteinase Activity		Neutral Proteinase Activity			Plasminogen ^b
	Total	Cathepsin D	Cathepsin B	Total	Cathepsin + PMLE	
Control	5.2 ± 0.3	4.7 ± 0.4	0.5 ± 0.4	0.24 ± 0.05	0.08 ± 0.02	0.10 ± 0.06
Low	5.4 ± 0.5	4.9 ± 0.4	0.5 ± 0.3	0.14 ± 0.02	0.05 ± 0.02	0.18 ± 0.09
Medium	6.3 ± 0.6	5.3 ± 0.4	1.0 ± 0.4	0.26 ± 0.07	0.13 ± 0.05	0.00 ± 0.07
High	8.6 ± 0.5 ^c	8.1 ± 0.5 ^c	0.5 ± 0.4	0.23 ± 0.03	0.28 ± 0.03 ^c	0.28 ± 0.13

^aMean ± SE (n = 6) proteinase activity as mg protein released per hour and normalized to the average

lung weight from control animals (mg/hr/g control lung).

^bNeutral proteinase activated by streptokinase.^cStatistically different from controls in multiple comparison t-tests of the means ($p < 0.05$ for the null hypothesis).

the activity was Cathepsin D. This increase probably related to a pulmonary inflammatory process resulting from the reaction of lung tissue to the dye. The lack of response of Cathepsin B suggested that little turnover of the pulmonary architecture had occurred.

Neutral proteinase activity in lung tissue was affected to a lesser degree than was acid proteinase. At the high levels, only plasminogen (stimulation of proteinase activity by streptokinase) and Cathepsin G-polymorphonuclear leucocyte elastase (PMNLE) were increased, and only to a modest degree. These findings suggested that mostly lung tissue acid proteolytic activity was increased, which is consistent with an influx of inflammatory cells.

Lung Connective Tissue Biochemistry

Total lung collagen and airway collagenous peptides were measured in six control rats and six rats exposed to the highest level (210 mg/m^3) of SY/SG for 4 weeks. Total collagen content of the lung and airway collagenous peptides did not increase or decrease in SY/SG-exposed rats as compared to control animals, as judged by a two-tailed Mann-Whitney U statistical test at a significance level of $p \leq 0.05$ (Table 23).

Hematology and Serum Clinical Chemistries

Clinical chemistry measurements were made on 12 control rats and 12 rats at each exposure concentration (Tables 12 and 24). Comparisons were made between each exposed group and the control group, using multiple comparison t-tests. Results showed alkaline phosphatase and total bilirubin were increased slightly to the same levels, but the normal glutamic pyruvic transaminase levels, along with a lack of histopathological findings, indicates no hepatic necrosis was present. Changes in the other clinical

TABLE 23

Total Collagen Levels in Control F344 Rats and Rats Exposed
to SY/SG Dye Mixture for 30 Days^a

	<u>Total Collagen Content</u>	
	<u>Lung Collagen</u> <u>(mg/g control lung)^c</u>	<u>Lavage Fluid Peptides^b</u> <u>(μg/g control lung)^c</u>
Control	12.0 \pm 1.0	72 \pm 17
SY/SG Exposed (High Level)	11.6 \pm 0.6	95 \pm 11

^aMean \pm SE (n = 6).

^bDetermined by analysis of bronchoalveolar lavage fluid.

^cMilligrams or micrograms of lung collagen or lavage fluid peptides,
respectively, normalized to the average lung weight from control animals.

No statistical differences were found using the Mann-Whitney U test

(p \geq 0.05 for both comparisons).

TABLE 24

Changes in Selected Clinical Chemistry Measurements
After Inhalation of SY/S6 Dye

Mean \pm SE (n = 12)

Parameter	Control	Exposure Concentration		
		Low	Medium	High
Total CO ₂ (mmoles/L)	24 \pm 1	27 \pm 1	27 \pm 1	27 \pm 1
Alkaline Phosphatase (IU/L)	85 \pm 3	104 \pm 4*	105 \pm 6*	106 \pm 9*
Total Bilirubin (mg/dL)	0.2 \pm 0.1	0.3 \pm 0.1*	0.3 \pm 0.1*	0.4 \pm 0.1*
Total Protein (g/dL)	5.5 \pm 0.1	6.2 \pm 0.1	6.2 \pm 0.1	6.1 \pm 0.2
Albumin (g/dL)	4.0 \pm 0.1	4.4 \pm 0.1*	4.4 \pm 0.1*	4.3 \pm 0.1
Albumin/Globulin	2.7 \pm 0.1	2.5 \pm 0.2	2.6 \pm 0.2	2.7 \pm 0.3
BUN (mg/dL)	20 \pm 1	42 \pm 9	40 \pm 8	31 \pm 6
Creatinine (mg/dL)	0.4 \pm 0.1	0.5 \pm 0.1*	0.6 \pm 0.1*	0.6 \pm 0.1*
Inorganic Phosphorus (mg/dL)	6.8 \pm 0.2	7.4 \pm 0.6	7.2 \pm 0.5	8.1 \pm 0.4*
Cholesterol	84 \pm 5	73 \pm 9	72 \pm 6	99 \pm 7
Glucose	164 \pm 10	181 \pm 4	186 \pm 7	159 \pm 4

*Statistically different from controls in multiple comparison t-tests of the means ($p < 0.05$ for the null hypothesis).

chemistry measurements were not considered to be of biological significance (Table 24).

Immunology (Skin Sensitivity)

The SY portion of SY/SG dye mixture is a known allergic sensitizer in humans.¹⁰ Therefore, experiments were conducted to evaluate if hypersensitive skin reactions occurred from repeated SY/SG exposures. Groups of eight control and eight high level exposed rats (equally divided by sex) were injected with 500 µg of SY/SG, a dose which does not cause irritation. In 4 rats from each group, dye injections were intradermal in the ear. The remaining 4 rats in each group were injected with 0.1 mL saline. Injections were completed within 24 hours after the end of the 4-week exposures. The reactions in the ears injected with dye and saline were evaluated at 15-30 minutes, 8, 24, and 48 hours after injections for the evaluation of Type I (IgE mediated), Type III (Arthus), and Type IV hypersensitivity. Results indicated no hypersensitivity reactions to have occurred in exposed rats as compared to controls. However, it must be noted that the rat is not an ideal animal model for studying skin sensitivities.

Histopathology

The histopathology evaluations were done at Experimental Pathology Laboratories, Inc., Herndon, VA. Based on early damage indicators in the lavage fluid, only tissues from control, medium, and high level exposure rats were examined. Routine 5-µm hematoxylin and eosin stained slides were prepared from lung, larynx, trachea, and nasal turbinates and cavity from the rats. Four sections of the rat nasal cavity were prepared as per protocol. In addition, the following slides from all rats were prepared: skin, tracheobronchial and popliteal lymph nodes, spleen, femur, heart, stomach,

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duodenum, cecum, colon, liver, pancreas, kidney, urinary bladder, epididymis, testis, prostate, uterus, ovary, adrenal, thyroid, brain, pituitary, eye, and gross lesions.

Microscopic examination revealed a mild reaction around the terminal airways of the lungs from animals exposed to the highest dose of the yellow-green dye mixture. This exposure-related lesion consisted of minimal to slight proliferation of foamy alveolar macrophages and minimal to slight hyperplasia of Type II pulmonary epithelial cells. In some animals, especially the males, there were a few scattered polymorphonuclear leukocytes. This reaction was observed around most of the terminal airways and was more obvious in males than in females. Although mild in the high-dose animals, it was also observed in some of those exposed to the medium dose. Exposure-related particles were not observed in the lungs. Most of the rats had minimal to slight proliferations of lymphoid cells in perivascular and peribronchiolar areas. This change was seen in controls as well as exposed animals.

In the tracheobronchial lymph nodes, clusters of reticuloendothelial cells with lymphoid hyperplasia were observed in the animals exposed to the highest concentration of the dye. Although phagocytized particles were not visible, the clusters of reticuloendothelial cells suggest that foreign particles have been moved from the lungs to the tracheobronchial lymph nodes.

A yellowish-brown pigment was seen below the respiratory epithelium of the nasal septum and turbinates which was apparently exposure related. This was seen only in the high level exposure group of rats for both sexes. No such pigments were found in the larynx, trachea or bronchi. Minimal to slight lymphoid infiltrates under the epithelium lining, the dorsal meatus or around

the nasolacrimal duct was also seen in some of the nasal cavities in all groups, including the controls. This was usually more noticeable in males than in females.

An isolated finding unrelated to treatment was mycotic rhinitis in one rat from the highest exposure group. The organism, visible microscopically, resembled *Aspergillus*. Other changes appeared to be spontaneous lesions in this group of rats, for example, dilated gastric mucosal glands, a renal infarct, and testicular atrophy.

In brief, a mild exposure-related lesion was observed around the terminal airways in the lungs of all rats exposed to the highest dose of solvent yellow-green dye mixture for four weeks. This exposure-related lesion, consisting of Type II pulmonary epithelial cell hyperplasia and proliferation of foamy alveolar macrophages, was more noticeable in rats exposed to the highest concentration of dye than in rats exposed to the medium concentration, and was also more apparent in males than in females. Lymphoid hyperplasia and clusters of reticuloendothelial cells, seen in the tracheobronchial lymph nodes, also appeared to be exposure-related. No exposure-related lesions were observed in sections of trachea, larynx, and nasal cavity. Changes in other organs appeared to be incidental and unrelated to treatment.

SUMMARY OF THE FOUR-WEEK REPEATED

INHALATION EXPOSURE OF RATS TO DYE AEROSOLS

Male and female F344 rats were exposed by inhalation to 10 ± 5 , 51 ± 10 or $230 \pm 30 \text{ mg/m}^3$ (mean \pm SD) of SY dye aerosol for 6 hr/day, 5 days/wk for 4 weeks. The average particle size of these aerosols ranged from 3.1 to 4.4 μm (mass median aerodynamic diameter). Following exposures, lung concentrations of Q1 were measured, which indicated that very little SY dye was retained in lungs and thus the amount deposited had cleared at a very rapid rate. Exposed animals were also evaluated by a number of parameters for changes in respiratory function, biochemical indications of lung tissue damage, changes in blood chemistries, immunological responses and histopathological lesions. Respiratory function measurements indicated trends consistent with emphysematous changes in the lungs of rats exposed to highest level of SY dye, but no histological evidence of emphysema was found. Longer exposure times at this concentration would be needed to confirm this effect. Increases in tissue acid proteinase activity were seen. The major increase was associated with the activity inhibited by leupeptin (i.e., "cathepsin B-like" activity). The increase is consistent with the turnover of pulmonary connective tissue, and thus with the larger and more compliant lungs of rats exposed to the SY dye material. No other adverse effects were observed in the various parameters measured in exposed animals as compared to control rats. The only toxicological observation that arose from this study was that, at the end of the exposure, both male and female rats exposed to the highest concentration of SY dye had significantly lower body weights than the other exposure groups of animals including controls. Therefore, it appears that the lowest SY inhalation exposure level that will result in pathological alterations is $\geq 230 \text{ mg/m}^3$ in this particular exposure regime.

Male and female F344 rats were also exposed by inhalation to 11 ± 5 , 49 ± 11 or $210 \pm 50 \text{ mg/m}^3$ (mean \pm SD) of SY/SG dye aerosol for 6 hr/day, 5 days/wk for 4 weeks. The average particle size of these aerosols ranged from 3.2 to 4.9 μm (mass median aerodynamic diameter). After exposures, it was found that the major component of the SY portion of this dye mixture (Q1) cleared from lungs at a very rapid rate, but that substantial amounts of the major component of the SG portion of this dye mixture (TA) remained in lungs after the exposure. As in the four-week exposure study to SY dye, rats exposed to the highest concentration of SY/SG dye mixture gained weight at a significantly slower rate than the other experimental groups of animals. Respiratory function measurements demonstrated minor changes in lung volume subdivisions, a reduction of gas exchange efficiency, and mild airflow obstruction in high level exposed animals. Analysis of bronchoalveolar lavage fluid showed a mild pulmonary inflammatory response to have occurred in high level SY/SG exposed rats. Exposure-related histopathological lesions consisted of Type II pulmonary epithelial cell hyperplasia and proliferation of foamy alveolar macrophages. These latter observations were more noticeable in rats exposed to the highest concentration of SY/SG dye than in the medium level exposed animals, and in males than in females. Lymphoid hyperplasia and clusters of reticuloendothelial cells were seen in the tracheobronchial lymph nodes, but only in the high level exposed animals. No other adverse effects were observed in the various parameters measured in exposed animals as compared to control rats. Therefore, it appears that the lowest SY/SG inhalation exposure level that will result in pathological alterations is $\geq 50 \text{ mg/m}^3$.

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GLOSSARY

Green component of yellow/green dye: green dye, solvent green, SG

Major compound in yellow dye: 2-(2'-quinoly1)-1,3-indandione, Q1

Major compound in green dye: 1,4-di-p-toluidinoanthraquinone, TA

Stock yellow dye: yellow dye, solvent yellow, SY

Stock yellow/green dye: yellow/green dye mix, SY/SG

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